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# Pathobiology of Asian Highly Pathogenic Avian Influenza H5N1 Virus Infections in Ducks

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SUMMARY. Ducks and other wild aquatic birds are the natural reservoir of type A influenza viruses, which normally are nonpathogenic in these birds. However, the Asian highly pathogenic avian influenza (HPAI) viruses have evolved from producing no disease or mild respiratory infections in ducks to some strains producing severe systemic disease and mortality. To further understand the pathogenicity of these strains in ducks, we studied the gross and histologic lesions and tissue distribution of viral antigen in 2- and 5-wk-old white Pekin ducks infected with different Asian-origin H5N1 AI viruses. Seven of eight 2-wk-old ducks inoculated with A/Egret/HK/757.2/02 developed acute disease, including severe neurological dysfunction and death. However, this virus killed only two of eight 5-wk-old ducks. Two additional viruses, A/Vietnam/1203/04 and A/Crow/Thailand/04, also produced high mortality in 2-wk-old ducks. Microscopic lesions and AI viral antigen were observed most frequently in the nasal cavity, brain, heart, adrenal glands, and pancreas. Another virus, A/Thailand PB/6231/04, killed three of eight 2-wk-old ducks but did not induce neurological signs. Furthermore, older ducks infected with this virus did not present clinical signs or gross lesions, and their tissues showed very few microscopic lesions. All the viruses studied established systemic infections in both younger and older ducks, with viral replication in tissues correlating with the severity of the clinical signs. The differences in mortality induced by HPAI H5N1 viruses in ducks are reflected in the pathological findings and antigen distribution in tissues. However, the observed differences in pathology between ducks infected at different ages is unclear and may be associated with a variety of factors including the virus strain, host immune response, host cell maturation, and capacity to support viral replication.

RESUMEN. Patobiología de las infecciones con el virus Asiático H5N1 de influenza aviar de alta patogenicidad en patos.

Los patos y otras aves acuáticas silvestres constituyen el reservorio natural de los virus de influenza aviar tipo A, los que normalmente son apatógenos en estas aves. Sin embargo, los virus Asiáticos de influenza aviar de alta patogenicidad han evolucionado desde ser apatógenos o producir infecciones respiratorias leves en patos hasta algunas cepas capaces de producir enfermedades sistémicas severas y mortalidad. Con la finalidad de entender mejor la patogenicidad de estas cepas en patos, se estudiaron las lesiones macroscópicas e histológicas y la distribución del antígeno viral en los tejidos de patos pequineses blancos de 2 y 5 semanas de edad infectados con diferentes virus H5N1 de influenza aviar originarios de Asia. Siete de ocho patos de dos semanas de edad inoculados con el virus A/Garza/HK/757.2/02 desarrollaron enfermedad aguda, incluyendo disfunción neurológica severa y muerte. Sin embargo, este virus solo mató dos de ocho patos de 5 semanas de edad. Dos virus adicionales, el virus A/Vietnam/1203/04 y el A/Cuervo/Tailandia/04, también produjeron alta mortalidad en patos de 2 semanas de edad. Las lesiones microscópicas y la presencia de antígeno viral de influenza aviar fueron observadas más frecuentemente en la cavidad nasal, cerebro, corazón, glándulas adrenales y páncreas. Otro virus, el A/Tailandia PB/6231/04, mató tres de ocho patos de 2 semanas de edad, pero no indujo signos neurológicos. Más aun, patos de más edad infectados con este virus no presentaron signos clínicos o lesiones macroscópicas y en sus tejidos se observaron muy pocas lesiones microscópicas. Todos los virus estudiados establecieron infecciones sistémicas tanto en los patos jóvenes como en los mayores, con replicación viral en tejidos en correlación con la severidad de los signos clínicos. Las diferencias en mortalidad inducida por los virus H5N1 de influenza aviar de alta patogenicidad en patos se reflejan en los hallazgos patológicos y en la distribución antigénica en tejidos. Sin embargo, las diferencias en la patología observadas entre patos infectados a diferentes edades no está clara y debe estar relacionada con una variedad de factores incluyendo la cepa, la respuesta inmune del huésped, el grado de madurez de las células del huésped y la capacidad de sustentar la replicación del virus.

Key words: avian influenza, ducks, H5N1 virus, immunohistochemistry, pathogenicity

Abbreviations: AGP = agar gel precipitin; AI = avian influenza; BHI = brain heart infusion; BSL-3 Ag = biosafety level-3 agriculture; dpi = days postinoculation; $EID_{50}$ = 50% egg infectious dose; HE = hematoxylin and eosin; HEPA = high efficiency particulate air; HPAI = highly pathogenic avian influenza; IHC = immunohistochemistry/chemical; IN = intranasal; MDT = mean time death; SEPRL = Southeast Poultry Research Laboratory

Highly pathogenic avian influenza (HPAI) viruses produce a severe, systemic disease with high mortality in chickens and other galliforme birds but usually do not cause clinical disease or death in ducks (33). Ducks and other wild aquatic birds are the natural host species and reservoir for influenza viruses (28,34). However, prior to the emergence of the Asian HPAI H5N1 viruses, only once had AI viruses been reported to cause the death of ducks, this occurring during an HPAI H7N1 virus outbreak in Italy in 1999–2000, where the death of Muscovy ducks was reported (6). The Asian HPAI H5N1 viruses have shown different ability to induce disease in ducks. The 1997–2001 HPAI H5N1 viruses either produced no lesions or produced a mild respiratory infection in ducks (6,24,26,29). The pathobiology of HPAI H5N1 viruses in ducks began to change in 2001 and to show wide variations in lesions and lethality (18,21,35). In late 2002, HPAI H5N1 outbreaks in two Hong Kong parks, Kowloon Park and Penfold Park, caused the death of several wild avian species including waterfowl (8). Experimentally these viruses caused a systemic infection in ducks, with high viral titers and pathology in multiple organs, particularly in the brain (21,29). In other studies HPAI H5N1 viruses isolated from duck meat imported into Japan from China also induced neurological signs in ducks but did not cause mortality (14). However, several isolates from 2003 and 2004 experimentally induced mortality in mallard ducks (29,30).

Previous pathogenesis studies using ducks of different ages resulted in variable results; viruses appeared to be less pathogenic for older ducks (D. Swayne, unpubl. data). To understand the diverse pathogenicity of HPAI H5N1 viruses in ducks and the role of age in the outcome of infection, we examined the effects of infection with different Asian-origin HPAI H5N1 viruses in 2- and 5-wk-old Pekin ducks. The purpose of this study was to identify the pathobiologic changes associated with Asian H5N1 AI viruses in ducks. Specifically we examined the severity and distribution of gross and microscopic lesions in experimentally infected ducks and identified the organs and cell types with AI virus replication.

## MATERIALS AND METHODS

**Viruses.** The H5N1 influenza viruses used in this study were the following: A/Vietnam/1203/04 (received courtesy of Kanta Subbarao, U.S. National Institutes of Health), A/Thailand PB/6231/04 and A/Crow/Thailand/04 (received courtesy of Chantanee Buranathai, Department of Livestock Development, Thailand), and A/Egret/HK/757.2/02 (kindly provided by Trevor Ellis, Agriculture Fisheries and Conservation Department, Hong Kong). Virus stocks were propagated for 24–30 hr in the allantoic cavity of embryonating chicken eggs at 37 C. Allantoic fluid from inoculated eggs was collected and diluted 1:300 in brain heart infusion (BHI) medium. Similarly, a sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI. Serial titrations were performed in embryonating eggs and incubated at 37 C for 24–30 hr. Subsequently allantoic fluid from eggs were harvested, and 50% egg infectious dose ($EID_{50}$) titers were determined by testing hemagglutination activity (32). Titration endpoints were calculated by the method of Reed and Muench (25). All four H5N1 viruses had high infectivity titers in eggs ($10^{6.0}$ $EID_{50}$/ml). All experiments using HPAI H5N1 viruses, including work with animals, were conducted using biosafety level-3 Ag (BSL-3 Ag) containment procedures (1), and all personnel were required to wear a powered air protection respirator with high efficiency particulate air (HEPA)–filtered air supply (3M™, St. Paul, MN).

**Experimental design.** *Pathogenicity studies in 2-wk-old ducks.* Two-week-old Pekin ducks (obtained from Ideal Poultry Breeding Farms, Cameron, TX) were intranasally (IN) inoculated to determine the pathogenesis of the four HPAI H5N1 viruses previously mentioned. Serum samples were collected from all ducks prior to inoculation to ensure that the birds were serologically negative for AIV as determined with the agar gel precipitin (AGP) test (2). Ducks were housed in self-contained isolation units (Mark 4, Controlled Isolation Systems, San Diego, CA) that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. Feed and water were provided *ad libitum.* General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (7).

Ducks were separated into a control group and four virus-inoculated groups. The control group contained five ducks; these were inoculated IN with 0.1 ml of the sham inoculum. Two control birds were euthanatized at 2 and 14 days postinoculation (dpi) (four birds total), and heart, lungs, bursa, thymus, adrenal gland, kidney, liver, spleen, skeletal muscle, proventriculus, gizzard, pancreas, intestine, bill, trachea, and brain tissue were collected in 10% buffered formalin for histopathologic evaluation. At 14 DPI, serum was collected from the three remaining control birds for AGP to ensure that controls remained serologically negative to AI virus.

The four virus-inoculated groups, each containing 10 birds, were inoculated IN with 0.1 ml of inoculum containing $10^6$ $EID_{50}$/ml of the viruses. Two birds from each group were euthanatized at 2 dpi to determine the extent of virus replication in tissues. The remaining eight birds were observed for signs of illness over a 14-day period during which clinical signs and weights were recorded. Oropharyngeal and cloacal swabs were collected from all ducks each day from 1 to 7 days and from two ducks per group on 10 and 14 dpi. Samples were stored frozen at −70 C, and titers of infectious virus were subsequently determined as previously described (5). Ducks that showed severe disease signs were euthanatized. Tissues for histopathologic examination were collected from euthanatized and select recently deceased birds as described for the control group. Serum and tissues were collected from all virus-inoculated birds alive at the termination of the experiment. Sample birds, moribund birds, and all birds remaining at the end of the 14-day period were euthanatized by the intravenous administration of sodium pentobarbital (100 mg/kg body weight).

*Pathogenicity studies in 5-wk-old ducks.* Five-week-old Pekin ducks (obtained from Murray McMurray Hatchery, Webster City, IA) were inoculated IN to determine the pathogenesis of A/Egret/HK/757.2/02 and A/Thailand PB/6231/04. These viruses were chosen based on the results obtained with the 2-wk-old ducks, the first virus causing severe disease and the second causing moderate disease. Housing and care conditions were the same as for 2-wk-old ducks. Ducks were separated into a control group and two virus-inoculated groups. The control group contained 10 ducks; these were inoculated IN with 0.1 ml of the sham inoculum. The two virus-inoculated groups, each containing 10 birds, were inoculated IN with 0.1 ml of inoculum containing $10^6$ $EID_{50}$/ml of the viruses. Two birds from each group were euthanatized at 2 dpi, and the remaining eight birds were observed for signs of illness. Oropharyngeal and cloacal swabs and tissue samples were collected and processed as described. Ducks that showed severe disease signs were euthanatized. Tissues for histopathologic examination were collected as previously described, and sera were collected from two virus-inoculated birds surviving at the termination of the experiment.

*Histopathology and immunohistochemistry (IHC).* Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (HE). A duplicate 4-μm section was IHC stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A 1:2000 dilution of a mouse-derived monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein (developed at Southeast Poultry Research Laboratory [SEPRL], USDA, Athens, GA) was applied and allowed to incubate for 2 hr at 37 C. The primary antibody was then detected by the application of biotinylated goat anti-mouse IgG secondary antibody using a biotin-streptavidin detection system (Supersensitive Multilink Immunodetection System, Biogenex). Fast Red TR (Biogenex) served as the substrate chromagen, and hematoxylin was used as a counterstain. All tissues were systematically screened for microscopic lesions. Lesions were scored as follows: − = no lesions; + = mild; ++ = moderate; +++ = severe lesions. The intensity of viral antigen staining in each section was scored as follows: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread staining.

## RESULTS

**Control groups.** No mortality was observed in the sham-inoculated control ducks. The ducks were active, eating and drinking normally, and gaining weight. Grossly and histologically, control birds lacked significant lesions. Control birds bled at 14 dpi were negative to antibodies to AIV as determined by AGP.

**Two-week-old virus-inoculated ducks.** The mortality induced by infection with the viruses evaluated is summarized in Table 1. Ducks inoculated with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 had similar high mortality rates, and the mean death time (MDT) was between 4.1 and 4.5 days, whereas only three out of eight ducks inoculated with A/Thailand PB/6231/04 died, and the MDT was 6.3 days. Virus was recovered from the cloaca and oropharynx from each of the four HPAI H5N1virus-inoculted groups. The oropharynx titers were consistently higher than the cloaca titers for all the viruses studied.

Table 1. Pathogenicity of Asian HPAI H5N1 viruses in ducks inoculated IN at 2 and 5 wk of age with $10^6$ $EID_{50}$/ml of virus. Mean $log_{10}$ titers, expressed as $EID_{50}$/milliliter, from oropharyngeal and cloacal swabs were sampled from three-to-five individual ducks on the days indicated. The limit of detection was $10^{0.9}$ $EID_{50}$/ml.

| Age at inoculation | Virus | Mortality[A] | Oral titers 3 dpi | Cloaca titers 3 dpi | Oral titers 5 dpi | Cloaca titers 5 dpi |
|---|---|---|---|---|---|---|
| 2 wk | A/Vietnam/1203/04 | 7/8 | 4.9 | 2.0 | 4.4 | 1.8 |
| | A/Crow/Thailand/04 | 8/8 | 4.4 | 1.9 | 4.9 | 1.4 |
| | A/Egret/HK/757.2/02 | 7/8 | 5.8 | 2.3 | 4.5 | 2.7 |
| | A/Thailand PB/6231/04 | 3/8 | 3.7 | 1.3 | 2.4 | 0.9 |
| 5 wk | A/Egret/HK/757.2/02 | 2/8 | 4.9 | 4.1 | 3.3 | 0.9 |
| | A/Thailand PB/6231/04 | 0/8 | 2.4 | 0.8 | 2.1 | — |

[A]Number of dead ducks/number of inoculated or exposed ducks.

Ducks inoculated with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 presented severe depression and anorexia as early as 2 dpi. The ducks were quiet and reluctant to move. Two-to-three ducks in each of these groups had cloudy eyes. All ducks displayed mild to severe neurological signs beginning at 3 dpi characterized by tremors, uncontrollable shaking, marked loss of balance, lack of coordination, tilted head, seizures, and paralysis (Figs. 1a,b). Both ducks that survived (one inoculated with A/Vietnam/1203/04 and the other inoculated with A/Egret/HK/757.2/02) continued to have mild neurological signs until the end of the experiment. These ducks ate and drank water, although they looked emaciated. Three ducks were euthanatized on days 3 and 4 because of the severity of their neurological signs (severe seizures) or they were so depressed that they couldn't eat or drink water. Some ducks died presenting only mild neurological signs; however, they were severely depressed. Ducks inoculated with A/Thailand PB/6231/04 presented minimal clinical signs. At 5 and and 8 dpi the ducks were mildly depressed and had lack of appetite. The rest of the days the ducks in this group ate well and seemed alert, although a negative effect on weight gain was observed during the course of the experiment (data not shown).

*Gross lesions.* In total, 28 ducks from the groups inoculated with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 were examined when euthanatized or found dead. The gross lesions observed were similar between these three groups, and dehydration, flaccid proventriculus, empty intestines, splenomegaly, and thymus atrophy were present in most. Also commonly observed were a nasal yellowish discharge that could be expressed from the nostrils (8/28), cyanotic bill and toes (7/28), dilated and flaccid heart with increased pericardial fluid (5/28), renomegaly and/or renal pallor and accentuated lobular surface architecture (3/28), congested, malacic brain (3/28), impacted proventriculus and gizzard with intense bile staining of the mucosa (2/28), and yellowish pancreas with petechia (1/28). Of the two ducks that survived, one had a swollen bill filled with sero-sanguinolent liquid, and the other had a flaccid, dilated heart. Both were emaciated.

Of the ducks inoculated with A/Thailand PB/6231/04 that were euthanatized or found dead, 4/5 had thick yellowish secretions that could be expressed from the nostrils; 4/5 had serous exudates in body cavities, such as the pericardial sac and coelom, and enlarged, flaccid hearts; 3/4 had flaccid proventriculus and enlarged spleens; 2/5 had swollen kidneys; and 1/5 had intense bile staining of the mucosa of the proventriculus and koilin lining of the gizzard.

*Histologic lesions.* The distribution and severity of histologic lesions and AI antigen staining are summarized in Tables 2 and 3. Representative images of the histologic findings and viral antigen distribution are included in Figs. 1c–h and 2a–d. The distribution of lesions was similar between ducks euthanatized and examined at 2 dpi and ducks found dead and examined, although the lesions were more severe and the antigen staining stronger and more widespread within tissues in the ducks found dead or euthanatized because of the severity of their clinical signs.

In the ducks inoculated with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02, the most significant lesions were found in the nasal cavity, brain, heart, pancreas, adrenal gland, and skeletal muscle. In the brain, randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema were observed. Focally extensive neuronal degeneration and necrosis and vacuolation of the neuropil were present and were most severe in the ducks sampled after found dead. In the heart, random multifocal to confluent myocardial degeneration to necrosis was observed. Minimal-to-mild inflammation was frequently associated with myocardial necrosis and hyalinized fibers. The inflammatory cell population consisted of lymphocytes, plasma cells, and macrophages. These lesions were more severe in ducks found dead. Mild-to-severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium occurred in all of the sampled ducks. The corticotrophic cells and less consistently the chromaffin cells of the adrenal gland had mild-to-moderate multifocal to confluent areas of vacuolar degeneration to necrosis. Skeletal muscle sampled from the thigh contained degeneration to necrosis of individual myofibers.

The prevailing lesions within the respiratory tract were confined to the nasal cavity, with mild-to-moderate necrotizing rhinitis and sinusitis, mucocellular exudates containing sloughed epithelial cells and heterophils, submucosal edema, glandular hyperplasia and epithelial changes including loss of cilia, vacuolar degeneration, and necrosis. The trachea presented mild degenerative changes of the overlying epithelium and mild lymphocytic infiltration in the submucosa. The lesions present in the lung were mild to moderate consisting of congestion and interstitial inflammation with mixed mononuclear cells. Mild necrotizing bronchitis with lumen cellular debris and mild fibrino airsacculitis were also observed.

The intestinal epithelium was only minimally to mildly affected, with mild inflammatory changes in the lamina propria. In all groups, the proventriculus and gizzard occasionally contained small foci of superficial heterophilic infiltration that was accompanied by mild epithelial necrosis. Minimal necrosis of scattered hepatocytes with sinusoidal histiocytosis was observed in the liver. The spleen, thymus, bursa, and mucosa-associated lymphoid tissue had moderate-to-severe lymphoid depletion ranging from apoptosis to necrosis in remaining lymphocytes. Both cortical and medullary regions of bursa and thymus were affected. At 2 dpi, active lymphocellular depletion, indicated by numerous pyknotic to karryorhectic lymphocytes, was observed in both tissues. Renal changes indicative of dehydration, such as minimal-to-mild dilatation of the distal

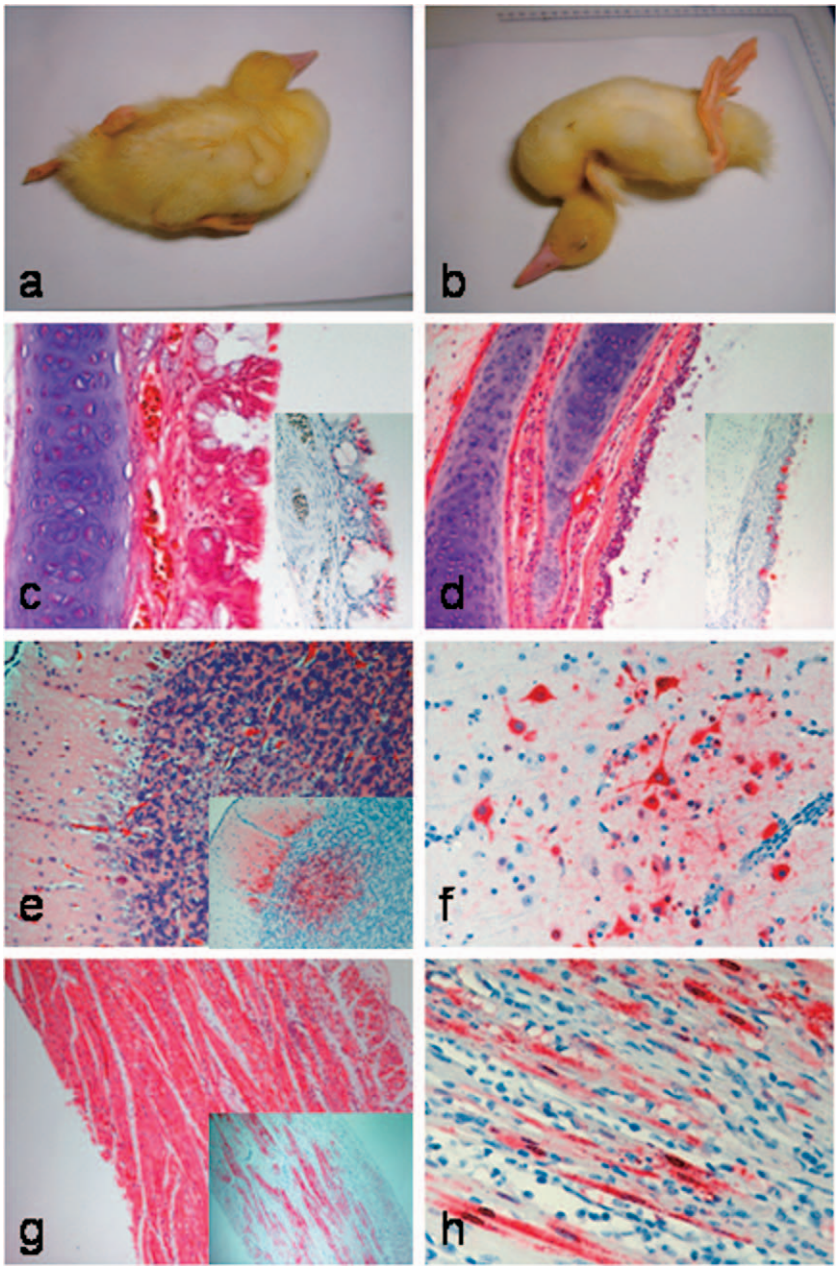


Fig. 1. (a,b) Two-week-old ducks showing severe neurological signs at 3 days after inoculated IN with A/Egret/HK/757.2/02. (c) Photomicrograph of the nasal epithelium of a 2-wk-old duck that died 3 days after inoculated IN with A/Crow/Thailand/04. Moderate necrotizing rhinitis, with submucosal congestion and edema, and glandular hyperplasia. Demonstration of viral antigen in the epithelial cells (insert). (d) Photomicrograph of the trachea of a 2-wk-old duck inoculated IN with A/Crow/Thailand/04 and found dead at 4 dpi. Degeneration and necrosis of the tracheal epithelium with mucocellular exudate containing sloughed epithelial cells. Viral antigen staining present in the epithelial cells (insert). (e) Photomicrograph of the cerebellum of a 2-wk-old duck inoculated with A/Egret/HK/757.2/02, 2 dpi. Vacuolation of the molecular and granular layers of the cerebellum with necrosis of the Purkinge neurons. Viral antigen present in the neuropil of both layers (insert). (f) Photomicrograph of the cerebrum of a 2-wk-old duck inoculated IN with A/Vietnam/1203/04 and found dead at 4 dpi. Strongly positive viral staining present in neurons. (g) Photomicrograph of the heart of a 2-wk-old duck inoculated IN with A/Crow/Thailand/04 and euthanatized at 4 dpi. Edema and myocardial degeneration and necrosis with mononuclear cell infiltration. Widespread viral staining present in the myocardial fibers (insert). (h) Photomicrograph of the heart of a 2-wk-old duck inoculated IN with A/Thailand PB/6231/04 and found dead at 5 dpi. Extensive intranuclear and intracytoplasmic viral antigen in degenerated and necrotic myocytes.

segments of nephrons, were observed in some of the ducks. Remaining organs lacked significant histopathologic lesions.

In the ducks inoculated with A/Thailand PB/6231/04 the most significant lesions were found in the nasal cavity, trachea, heart, adrenal gland, and pancreas. In general, the lesions were similar but milder compared to the ducks inoculated with the other three strains, but as opposed to these, no or very mild lesions were present in the brain.

*IHC.* In all four groups studied, viral antigen staining was present in multiple organs (Tables 2, 3, Figs. 1c–h, 2a–d). In general, there was a strong correlation between the demonstration of viral antigen and the identification of histologic lesions. However, viral antigen

Table 2. Distribution of histologic lesions and viral antigen resultant from intranasal inoculation of 2-wk-old ducks with A/Vietnam/1203/04, A/Thailand PB/6231/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 AI viruses, at 2 dpi.

| | A/Vietnam/1203/04 | | A/Thailand PB/6231/04 | | A/Crow/Thailand/04 | | A/Egret/HK/757.2/02 | |
|---|---|---|---|---|---|---|---|---|
| Tissue[A] | HE[B] | IHC[C] | HE | IHC | HE | IHC | HE | IHC |
| Nasal cavity | ++ | + | + | + | ++ | + | ++ | + |
| Trachea | + | + | ++ | ++ | + | + | − | + |
| Lung | + | + | ++ | + | + | + | ++ | + |
| Heart | ++ | +++ | + | ++ | ++ | +++ | + | ++ |
| Brain | ++ | ++ | + | − | +++ | +++ | ++ | + |
| Adrenal gland | +++ | +++ | − | − | + | ++ | − | − |
| Enteric tract | − | − | − | − | + | − | + | − |
| Pancreas | +++ | ++ | ++ | ++ | +++ | ++ | + | + |
| Liver | − | − | ++ | − | − | − | + | − |
| Kidney | − | + | − | − | + | + | − | − |
| Spleen | − | + | + | + | − | − | + | − |
| Bursa | ++ | − | − | − | ++ | − | + | + |
| Thymus | ++ | ++ | + | − | − | − | + | + |
| Skeletal muscle | ++ | ++ | − | − | + | ++ | − | + |
| Gizzard | − | − | − | − | − | − | − | − |
| Proventriculus | − | − | − | − | − | − | − | − |

[A]Tissues collected from two ducks at 2 dpi.
[B]HE, histologic lesions: − = no lesions; + = mild; ++ = moderate; +++ = severe.
[C]IHC staining: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

distribution was more widespread within tissues than the associated histologic lesions. Viral antigen was closely associated with the observed lesions in the pancreatic acinar epithelium, neurons and glial cells of the brain, epithelium of the nasal cavity and trachea, fragmented cardiac and skeletal myofibers, and adrenal corticotrophic cells. In lymphoid organs, viral antigen was identified only in resident and infiltrating phagocytes and not in apoptotic lymphocytes. Vascular endothelium was consistently negative for the presence of viral antigen. The staining within positive cells was always present in the nucleus and frequently in the cytoplasm, where it had a granular appearance.

At 2 dpi, viral antigen was detected in the epithelium of the nasal cavity and trachea, phagocytes of the lung, air sac epithelium, neurons and glial cells of the brain, cardiac and skeletal myocytes, adrenal corticotrophic cells, renal tubular epithelium, bursal phagocytes, medullary epithelium and tingible body macrophages of the thymus, and pancreatic acinar epithelium. Viral antigen staining was rare or infrequent in renal tubular epithelium and phagocytes of the spleen. In ducks that died or were euthanatized because of severe clinical signs, the viral staining distribution was more widespread and intense, with viral antigen also found in the epithelium and autonomic ganglia of the enteric tract, Kupfer cells of the liver, smooth muscle of the gizzard, and phagocytic cells of the proventriculus.

In ducks inoculated with A/Thailand PB/6231/04, viral antigen staining was less common than observed with the other three strains. At 2 dpi, no viral antigen was present in the brains and was infrequently found in tissues from ducks that died. In the ducks that died, viral antigen staining was found widespread in the heart, associated with the lesions observed in the myocardium.

**Five-week-old virus-inoculated ducks.** IN administration of A/Egret/HK/757.2/02 influenza virus resulted in the death of 2/8 ducks with a mean death time of 5 days after inoculation (Table 1). IN administration of A/Thailand PB/6231/04 did not induce mortality in 5-wk-old ducks. Virus was recovered from the oropharynx of ducks from both H5N1 AI virus-infected groups at 3 dpi, and from the cloaca of ducks inoculated with A/Egret/HK/757.2/02 at 5 dpi (Table 1). No clinical signs were observed in the 5-wk-old ducks inoculated with A/Thailand PB/6231/04, although a mild negative effect on weight was observed between 3 and 6 dpi (data not shown). Ducks inoculated with A/Egret/HK/757.2/02 presented loss of appetite, depression, and weakness at 4 and 5 dpi. After 6 dpi these ducks resumed eating normally. Also, 4/8 of the ducks inoculated with A/Egret/HK/757.2/02 displayed neurological signs beginning at 4 dpi characterized by tremors, lack of coordination, intermittent head shake, and head tilt. One of these ducks was euthanatized at 5 dpi because of severe seizures and paralysis. Another duck, presenting only mild neurological signs, was found dead at 6 dpi. The other 2 ducks that had neurological signs recuperated from the depression and resumed eating in spite of being uncoordinated and having irregular head movements. Two of the ducks that did not present neurological signs remained mildly depressed until the end of the experiment even though they continued to eat well. Three ducks had cloudy eyes between 3 and 6 dpi, and petechial hemorrhages were observed in the foot web of four of the most affected ducks between 4 and 8 dpi.

*Gross lesions.* Two ducks from each of the virus-inoculated groups and from the control group were euthanatized and examined at 2 dpi and at the end of the experiment (14 dpi). No gross lesions were observed in any of the ducks inoculated with A/Thailand PB/6231/04. The two ducks inoculated with A/Egret/HK/757.2/02 and examined at 2 dpi had enlarged flaccid hearts and proventriculi, congested intestines with watery contents, and a thick yellowish nasal discharge when pressure was applied to the nostrils. The two ducks from this group that were or euthanatized or found dead also had increased pericardial fluid, thickened air sacs, congestion and edema of the lungs, parenchymal pallor and accentuated lobular surface architecture of the kidney, thymus atrophy, and pale, malacic brains. The duck that was found dead also had a few hemorrhagic spots, 2–6 mm in diameter, on the dorsal and ventral surfaces of the beak and on the foot webbing. The two ducks from this group that presented mild neurological signs and were examined at the end of the experiment also had enlarged flaccid hearts and pale areas in the brain.

*Histologic lesions.* The distribution and severity of histologic lesions and AI antigen staining are summarized in Tables 4 and 5. In the ducks inoculated with A/Thailand PB/6231/04 the most significant lesions were found in the nasal cavity, trachea, lung,

Table 3. Distribution of histologic lesions and viral antigen resultant from intranasal inoculation of 2-wk-old ducks with A/Vietnam/1203/04, A/Thailand PB/6231/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 AI viruses. Ducks were found dead or were euthanatized because of the severity of the clinical signs.

| | A/Vietnam/1203/04 | | A/Thailand PB/6231/04 | | A/Crow/Thailand/04 | | A/Egret/HK/757.2/02 | |
|---|---|---|---|---|---|---|---|---|
| Tissue[A] | HE[B] | IHC[C] | HE | IHC | HE | IHC | HE | IHC |
| Nasal cavity | ++ | ++ | ++ | + | +++ | +++ | ++ | + |
| Trachea | ++ | +++ | + | + | +++ | +++ | + | + |
| Lung | ++ | + | + | + | ++ | + | ++ | + |
| Heart | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Brain | +++ | +++ | + | + | +++ | +++ | +++ | +++ |
| Adrenal gland | +++ | +++ | ++ | ++ | +++ | +++ | ++ | +++ |
| Enteric tract | +++ | ++ | + | + | ++ | ++ | ++ | + |
| Pancreas | +++ | +++ | + | + | +++ | +++ | ++ | +++ |
| Liver | + | ++ | − | + | + | + | + | + |
| Kidney | + | ++ | − | + | ++ | ++ | + | + |
| Spleen | + | + | − | − | − | − | + | + |
| Bursa | +++ | ++ | ++ | − | +++ | ++ | +++ | + |
| Thymus | ++ | ++ | + | + | ++ | ++ | ++ | ++ |
| Skeletal muscle | ++ | ++ | + | ++ | +++ | +++ | ++ | ++ |
| Gizzard | + | + | − | − | − | − | + | + |
| Proventiculus | ++ | ++ | + | + | ++ | ++ | + | + |

[A]Tissues collected from two-to-seven birds per group.
[B]HE, histologic lesions: − = no lesions; + = mild; ++ = moderate; +++ = severe.
[C]IHC staining: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

and pancreas of the ducks examined at 2 dpi. These lesions consisted of mild-to-moderate necrotizing rhinitis, mild-to-moderate lymphohystiocytic infiltration in the lungs, mild enteritis, and mild necrosis of pancreatic acinar epithelium. The only lesions present in the two ducks examined at 14 dpi were found in the pancreas, liver, and spleen and consisted of mild focal necrosis of the pancreatic acinar epithelium, mild focal coagulative necrosis of the hepatic parenchyma, and lymphoid depletion of the periellipsoidal and periarteriolar sheaths of the spleen.

In the ducks inoculated with A/Egret/HK/757.2/02, the most significant lesions were found in the nasal epithelium, brain, heart, and pancreas of euthanatized or dead ducks. Ducks examined at 2 dpi presented mild-to-moderate lesions in the nasal cavity, trachea, lung, intestinal tract, pancreas, liver, spleen, and bursa. No lesions were observed in the heart or brain of these ducks at this time point. Severe lesions were observed in the nasal cavity, heart, brain, pancreas, and bursa of the duck euthanatized and the one found dead. Mild-to-moderate lesions were observed in the trachea, lung, adrenal gland, enteric tract, liver, kidney, and spleen. The two ducks examined at 14 dpi had severe lesions in the heart and brain, moderate lesions in the kidney and lung, and mild lesions in the nasal cavity, liver, and proventriculus. Remaining organs lacked significant histologic lesions. Lesions in the brain of ducks examined at 2 dpi consisted in randomly scattered foci of malacia with gliosis, mild lymphoplasmacytic perivascular cuffs, and mild perivascular edema. In addition, severe lymphocytic infiltration, vacuolation of the neuropil, and the presence of eosinophylic concretions were observed in the brains of ducks examined at 14 dpi (Figs. 2e,f). In the heart, mild-to-moderate mononuclear inflammation was frequently associated with myocardial necrosis and hyalinized fibers (Fig. 2g). In the pancreas, moderate-to-severe multifocal cellular swelling and necrosis of the pancreatic acinar epithelium occurred in most sampled ducks. The primary lesions within the respiratory tract of ducks examined at 2 dpi were found in the nasal cavity and trachea and consisted of mild sinusitis and tracheitis. However, the two ducks examined at 14 dpi presented moderate lymphohystiocytic pneumonia (Fig. 2h). The euthanatized and dead ducks also presented moderate inflammatory changes in the lamina propria of the intestine and sloughing of the intestinal epithelium. Mild focal degeneration and necrosis of the adrenal corticotrophic cells were observed in the adrenal glands. The bursa had moderate to severe lymphoid depletion ranging from apoptosis to necrosis in remaining lymphocytes. Both cortical and medullary regions of bursa were affected. Renal changes indicative of dehydration, such as minimal-to-mild dilatation of the distal segments of nephrons, were observed in some ducks. Lesions in the spleen and liver were similar to those described for the ducks inoculated with A/Thailand PB/6231/04.

*IHC.* As observed with the 2-wk-old ducks, there was a strong correlation between the presence of viral antigen and the identification of histologic lesions. Viral antigen was detected in the epithelium and submucosa of the nasal cavity, in phagocytic leukocytes in the propria of the nasal cavity, trachea, and lung, and in the pancreatic acinar epithelium in ducks inoculated with either one of the viruses at 2 dpi. Viral antigen was also detected in cardiac myocytes and bursal phagocytes, in ducks inoculated with A/Egret/HK/757.2/02, and in skeletal myocytes in ducks inoculated with A/Thailand PB/6231/04 at 2 dpi. In the duck euthanatized and the one found dead, widespread viral staining was found in the nasal epithelium, neurons and glial cells of the brain, and cardiac myocytes. Staining was present also in the pancreatic acinar epithelium, and infrequently in the trachea, lung, adrenal gland, liver, spleen, and bursa. At 14 dpi, minimal viral staining was observed in the lung, heart, and brain in ducks inoculated with A/Egret/HK/757.2/02. No viral staining was detected in any of the tissues of ducks inoculated with A/Thailand PB/6231/04 at 14 dpi.

## DISCUSSION

To examine the outcome of infection with AI viruses, Pekin ducks were IN inoculated at 2 and 5 wk of age with different Asian-origin HPAI H5N1 viruses, and their organs subjected to pathologic and IHC analysis. Two-week-old ducks were used in this study following SEPRL's standard protocol for studying and comparing the

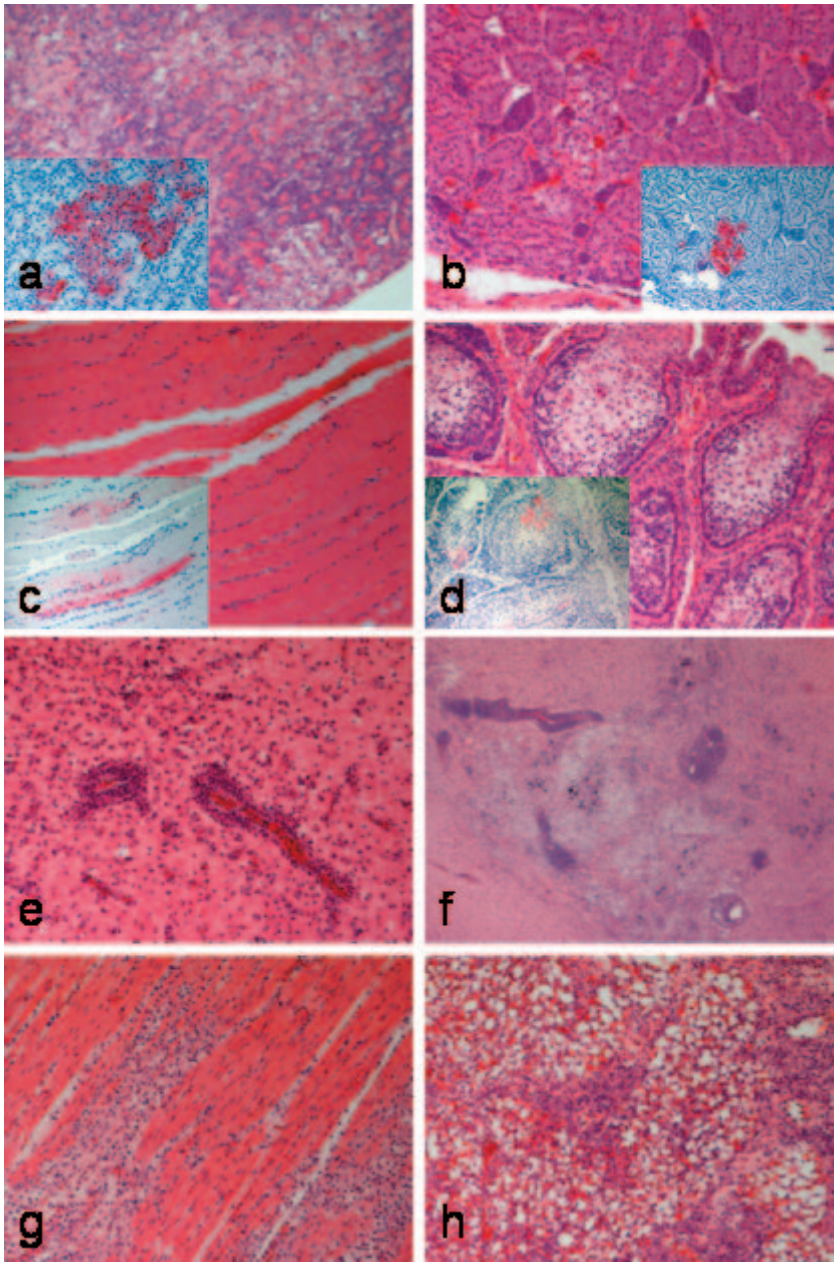


Fig. 2. (a) Photomicrograph of the pancreas of a 2-wk-old duck inoculated with A/Crow/Thailand/04, 2 dpi. Severe multifocal to confluent vacuolar degeneration to necrosis of pancreatic acinar epithelium with viral staining in the pancreatic acinar cells (insert). (b) Photomicrograph of the adrenal gland of a 2-wk-old duck inoculated IN with A/Vietnam/1203/04, 2 dpi. Focal necrosis of the corticotrophic cells of the adrenal gland with viral staining also in these cells (insert). (c) Photomicrograph of skeletal muscle of a 2-wk-old duck inoculated IN with A/Crow/Thailand/04 and euthanatized at 4 dpi. Hyalinization of skeletal myofibers with staining of the myocardial cells (insert). (d) Photomicrograph of the bursa of Fabricius of a 2-wk-old duck inoculated IN with A/Vietnam/1203/04 and found dead at 4 dpi. Lymphoid depletion of the bursa follicles. Viral antigen staining in intrafollicular macrophages (insert). (e) Photomicrograph of brain of a 5-wk-old duck inoculated with A/Egret/HK/757.2/02 that died with neurological signs, 14 dpi. Perivascular lymphoplasmacytic cuffs, gliosis, and vacuolar degeneration in the cerebrum. (f) Photomicrograph of the brain of a 5-wk-old duck inoculated with A/Egret/HK/757.2/02 that presented neurological signs, 14 dpi. Severe perivascular lymphoplasmacytic cuffs, lymphoplasmacytic infiltration of the neuropil, gliosis, malacia, and the presence of basophilic concretions in the cerebrum. (g) Photomicrograph of the heart of a 5-wk-old duck inoculated with A/Egret/HK/757.2/02 and found dead at 5 dpi. Severe multifocal mononuclear infiltration. (h) Photomicrograph of the lung of a 5-wk-old duck inoculated with A/Egret/HK/757.2/02 and found dead at 5 dpi. Moderate lymphoplasmacytic interstitial pneumonia.

pathogenicity of different AI viruses in ducks. Five-week-old ducks were the oldest ducks that could be used following the same protocol, given the length of the experiment and the ability of housing them adequately in the isolators.

The presence of microscopic lesions and AI viral antigen staining in tissues correlated with the severity of the clinical signs and the mortality observed in inoculated ducks. Two-week-old ducks infected with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 showed similar clinical signs, consisting of severe depression, neurological signs, and high mortality. The older ducks inoculated with one of these viruses, A/Egret/HK/757.2/02, exhibited only moderate mortality and less frequent neurological

Table 4. Distribution of histologic lesions and viral antigen resultant from IN inoculation of 5-wk-old ducks with A/Egret/HK/757.2/02 at 2 and 14 dpi or that died.

| | A/Egret/HK/757.2/02 at 2 dpi | | A/Egret/HK/757.2/02 at 14 dpi | | A/Egret/HK/757.2/02 euthanatized or dead (5 and 6 dpi) | |
|---|---|---|---|---|---|---|
| Tissue[A] | HE[B] | IHC[C] | HE | IHC | HE | IHC |
| Nasal cavity | ++ | ++ | + | − | +++ | +++ |
| Trachea | ++ | + | − | − | + | + |
| Lung | ++ | ++ | ++ | + | + | + |
| Heart | − | + | +++ | + | +++ | +++ |
| Brain | − | − | +++ | + | +++ | +++ |
| Adrenal gland | − | − | − | − | + | + |
| Enteric tract | + | − | − | − | ++ | − |
| Pancreas | ++ | + | − | − | +++ | ++ |
| Liver | + | − | + | − | + | + |
| Kidney | − | − | ++ | − | + | − |
| Spleen | + | − | − | − | + | + |
| Bursa | + | + | − | − | +++ | + |
| Thymus | − | − | − | − | − | − |
| Skeletal muscle | − | − | − | − | − | − |
| Gizzard | − | − | − | − | − | − |
| Proventiculus | − | − | + | − | − | − |

[A]Tissues collected from two birds per group at 2 and 14 dpi and from two birds that died at 5 and 6 dpi.
[B]HE, histologic lesions: − = no lesions; + = mild; ++ = moderate; +++ = severe.
[C]IHC staining: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

signs. The histologic lesions observed in tissues collected from the 2-wk-old ducks were similar, with the upper respiratory tract, brain, heart, pancreas, and adrenal glands the organs most consistently affected. The histologic lesions in the ducks that died or were euthanatized were more severe and involved other organs including intestines, bursa, thymus, and skeletal muscle. The histologic lesions observed in the tissues collected from ducks infected at 5 wk of age with A/Egret/HK/757.2/02 at 2 dpi were similar but milder than those observed in 2-wk-old ducks. However, moderate-to-severe lesions were present in heart and brain of the ducks that died or were examined at 14 dpi. The severe lymphocytic infiltration observed in these organs accounts for the increased lesion scores and points out a difference between older and younger ducks in their response to AI infection. Age-related outcomes of infection in ducks has been observed with other viruses, and proposed models for the effect of age include both increasing maturation of the immune system with age and a link between host cell maturation and the capacity to support viral replication (13).

Table 5. Distribution of histologic lesions and viral antigen resultant from IN inoculation of 5-wk-old ducks with A/Thailand PB/6231/04, at 2 and 14 dpi.

| | A/Thailand PB/6231/04 at 2 dpi | | A/Thailand PB/6231/04 at 14 dpi | |
|---|---|---|---|---|
| Tissue[A] | HE[B] | IHC[C] | HE | IHC |
| Nasal cavity | ++ | ++ | − | − |
| Trachea | − | + | − | − |
| Lung | ++ | + | − | − |
| Heart | − | − | − | − |
| Brain | − | − | − | − |
| Adrenal gland | − | − | − | − |
| Enteric tract | + | − | − | − |
| Pancreas | + | + | ++ | − |
| Liver | − | − | + | − |
| Kidney | − | − | − | − |
| Spleen | − | − | + | − |
| Bursa | − | − | − | − |
| Thymus | − | − | − | − |
| Skeletal muscle | − | + | − | − |
| Gizzard | − | − | − | − |
| Proventiculus | − | − | − | − |

[A]Tissues collected from two birds per group at 2 and 14 dpi.
[B]HE, histologic lesions: − = no lesions; + = mild; ++ = moderate; +++ = severe.
[C]IHC staining: − = no antigen staining; + = infrequent; ++ = common; +++ = widespread.

The death of ducks inoculated with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 was most likely associated with neurological damage. The virus may reach the brain through the blood or cranial nerves (12,14,19). This capacity to effectively obtain entry to and replicate within the brain is likely to be a strong determinant in the induction of morbidity and mortality in ducks and other avian species (22). Two- and 5-wk-old ducks inoculated with A/Thailand PB/6231/04 did not present neurological signs; however, 3/8 of the younger ducks died. Ducks infected with this virus presented no or only mild microscopic lesions in the brain, which explains the lack of neurologic signs. The 2-wk-old ducks infected with this virus most likely died from cardiac failure; this is supported by the gross and histologic lesions observed in the heart of the dead ducks.

To examine the tissue tropism of the viruses studied, collected tissues were subjected to IHC analysis using an antiserum against the AI viral nucleoprotein. AI virus antigen was observed in parenchymal cells with the predominate outcome being cell death as direct result of viral activity in these cells. Viral antigen staining in tissues collected from 2-wk-old ducks infected with A/Vietnam/1203/04, A/Crow/Thailand/04, and A/Egret/HK/757.2/02 was very similar and was present in multiple organs, indicating a systemic infection with these viruses. Viral staining was also present in tissues from 2-wk-old ducks inoculated with A/Thailand PB/6231/04; however, staining was less common, especially in the brain. Viral antigen staining in tissues from the 5-wk-old ducks infected with A/Egret/HK/757.2/02 or A/Thailand PB/6231/04 was also present in internal organs; however, the staining was less common or almost

nonexistent, especially in tissues from ducks inoculated with A/Thailand PB/6231/04.

Lesions indicative of vascular damage such as severe pulmonary edema, congestion, hemorrhage, and microtrombosis of capillaries, as well as viral staining of the vascular endothelium, consistently observed in chickens and other gallinaceous species (23,34) were not observed in ducks. However, the viruses studied demonstrated pantropic potential in ducks, with similar virus localization in the pancreas, central nervous system, adrenal glands, and myocardium as reported for other avian species (3,10,16,23,24,4,15,18,20,31). Localization of these viruses in the parenchyma of organs such as liver, intestinal tract, kidneys, and skeletal muscle was less consistent in ducks, indicating that virus distribution is dependent on particular host factors (9,23).

The increased capacity of Asian HPAI H5N1 viruses to cause systemic infections in ducks was clearly evident when in late 2002, H5N1 AI outbreaks in two Hong Kong parks, Kowloon Park and Penfold Park, caused the death of many resident avian species, including waterfowl (8). At Kowloon Park about 40% of the 199 waterfowl showed central nervous system involvement including depression, paresis, paralysis with or without tremors, and an intermittent head shake or an unusual head tilt. Interestingly, in this study, about one-quarter of the 80 ducks showing nervous symptoms recovered over time. Marked congestion and edema in lungs, congested brain with multifocal nonsupurative meningo-encephalitis, tracheitis, small foci of hepatic necrosis, and multifocal pancreatic necrosis were the most common lesions observed. IHC staining for viral antigen was positive on lungs and brains (8). Experimental infection studies using a goose HPAI H5N1 isolate from Penfold Park confirmed the neurotropism and broad tissue infectivity of these new H5N1 viruses (29), and an egret isolate (A/Egret/HK/757.2/02) also from this outbreak was found to be highly lethal for ducks; high titers of virus was detected in the lung, kidney, muscle and brain of birds inoculated with this strain (21). A great variation in pathogenicity among the circulating Asian H5N1 viruses has since been documented. Ducks inoculated with 23 different HPAI H5N1 viruses isolated in Asia between 2003 and 2004 showed different pathology, these viruses varying from nonpathogenic to highly lethal (11,30). Two Japan HPAI virus isolates, A/chicken/Yamaguchi/7/04 and A/duck/Yokohama/aq-10/03, replicated efficiently in multiple organs of infected 5-wk-old ducks; however, only the ducks infected with Dk/Yokohama/03 showed neurological signs, the virus growing in multiple organs more rapidly, with considerable titers of virus detected in the brain (14). These results are similar to what was also observed in this study, in that there are differences in pathogenicity among the Asian HPAI H5N1 viruses. It is important to point out that experimental results obtained by inoculation of AI viruses in ducks don't necessarily mimic what is observed in the field, in both domestic and wild ducks (8,17,27). Other factors, such as concomitant infections with other infectious agents, environmental factors, amount of viral exposure, and, as we demonstrated in this study, age of the ducks at infection, all interact to produce disease and mortality in ducks. This also explains the differences in gross and histologic lesions observed in ducks exposed naturally or experimentally to the virus, as, for example, the more severe lesions observed in the lungs of ducks from the park outbreaks in Hong Kong, and ducks from grazing systems or backyard flocks in Thailand (8,27).

In conclusion, the Asian HPAI H5N1 viruses have changed in their ability to cause disease in ducks; however, clinical signs and mortality depend on various factors including the strain of the virus, age of the ducks at exposure, and environmental factors including management. The level of viral replication in tissues, especially in brain and heart, determine the manifestation of clinical disease and mortality in AI-infected ducks.

## REFERENCES

1. Barbeito, M. S., G. Abraham, M. Best, P. Cairns, P. Langevin, W. G. Sterritt, D. Barr, W. Meulepas, J. M. Sanchez-Vizcaino, and M. Saraza. Recommended biocontainment features for research and diagnostic facilities where animal pathogens are used. First International Veterinary Biosafety Workshop. Rev. Sci. Tech. Off. Int. Epizoot. 14:873–887. 1995.

2. Beard, C. W. Avian influenza antibody detection by immunodiffusion. Avian Dis. 14:337–341. 1970.

3. Capua, I., S. Marangon, L. Selli, D. J. Alexander, D. E. Swayne, M. D. Pozza, E. Parenti, and F. M. Cancellotti. Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997–January 1998. Avian Pathol. 28:455–460. 1999.

4. Capua, I., F. Mutinelli, C. Terregino, G. Cattoli, R. J. Manvell, and F. Burlini. Highly pathogenic avian influenza (H7N1) in ostriches farmed in Italy. Vet. Rec. 146:356. 2000.

5. Cauthen, A. N., D. E. Swayne, S. Schultz-Cherry, M. L. Perdue, and D. L. Suarez. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J. Virol. 74:6592–6599. 2000.

6. Chen, H., G. Deng, Z. Li, G. Tian, Y. Li, P. Jiao, L. Zhang, Z. Liu, R. G. Webster, and K. Yu. The evolution of H5N1 influenza viruses in ducks in southern China. Proc. Natl. Acad. Sci. U S A 101:10452–10457. 2004.

7. Craig, J. V., W. F. Dean, G. B. Havenstein, K. K. Kruger, K. E. Nestor, G. H. Purchase, P. B. Siegel, and G. L. van Wicklen. Guidelines for poultry husbandry. In: Guide for the care and use of agricultural animals in agricultural research and teaching. Federation of American Societies of Food Animal Sciences, Savoy, IL. pp. 55–66. 1999.

8. Ellis, T. M., R. B. Bousfield, L. A. Bissett, K. C. Dyrting, G. S. Luk, S. T. Tsim, K. Sturm-Ramirez, R. G. Webster, Y. Guan, and J. S. Malik Peiris. Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol. 33: 492–505. 2004.

9. Hooper, P. T., G. W. Russell, P. W. Selleck, and W. L. Stanislawek. Observations on the relationship in chickens between the virulence of some avian influenza viruses and their pathogenicity for various organs. Avian Dis. 39:458–464. 1995.

10. Hooper, P., and P. Selleck. Pathology of low and high virulent influenza virus infections. In: Proc. Fourth International Symposium on Avian Influenza. D. E. Swayne and R. D. Slemons, eds. American Association of Avian Pathologists. Kennett Square, PA. 4:134–141. 1997.

11. Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc. Natl. Acad. Sci. U S A 102:10682–10687. 2005.

12. Iwasaki T., S. I., H. Nishimura, Y. Sato, M. Tashiro, T. Hashikawa, and T. Kurata. Productive infection in the murine central nervous system with avian influenza virus A (H5N1) after intranasal inoculation. Acta Neuropathol. 108:485–492. 2004.

13. Jilbert, A. R., J. A. Botten, D. S. Miller, E. M. Bertram, P. M. Hall, J. Kotlarski, and C. J. Burrell. Characterization of age- and dose-related outcomes of duck hepatitis B virus infection. Virology 244:273–282. 1998.

14. Kishida, N., Y. Sakoda, N. Isoda, K. Matsuda, M. Eto, Y. Sunaga, T. Umemura, and H. Kida. Pathogenicity of H5 influenza viruses for ducks. Arch. Virol. 150:1383–1392. 2005.

15. Kobayashi, Y., T. Horimoto, Y. Kawaoka, D. J. Alexander, and C. Itakura. Neuropathological studies of chickens infected with highly pathogenic avian influenza viruses. J. Comp. Pathol. 114:131–147. 1996.

16. Kobayashi Y., T. H., Y. Kawaoka, D. J. Alexander, and C. Itaruka. Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. Avian Pathol. 25:285–304. 1996.

17. Kwon Y.-K., S.-J. J., M.-C. Kim, H.-W. Sung, Y.-J. Lee, J.-G. Choi, E.-K. Lee, and J.-H. Kim. Highly pathogenic avian influenza (H5N1) in the

commercial domestic ducks of South Korea. Avian Pathol. 34:367–370. 2005.

18. Lee, C. W., D. L. Suarez, T. M. Tumpey, H. W. Sung, Y. K. Kwon, Y. J. Lee, J. G. Choi, S. J. Joh, M. C. Kim, E. K. Lee, J. M. Park, X. Lu, J. M. Katz, E. Spackman, D. E. Swayne, and J. H. Kim. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. J. Virol. 79:3692–702. 2005.

19. Matsuda K., C. D. P., Y. Sunden, T. Kimura, K. Ochiai, H. Kida, and T. Umemura. The vagus nerve is one route of transneural invasion for intranasally inoculated influenza in mica. Vet. Pathol. 41:101–107. 2004.

20. Mo, I. P., M. Brugh, O. J. Fletcher, G. N. Rowland, and D. E. Swayne. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. Avian Dis. 41:125–136. 1997.

21. Nguyen, D. C., T. M. Uyeki, S. Jadhao, T. Maines, M. Shaw, Y. Matsuoka, C. Smith, T. Rowe, X. Lu, H. Hall, X. Xu, A. Balish, A. Klimov, T. M. Tumpey, D. E. Swayne, L. P. Huynh, H. K. Nghiem, H. H. Nguyen, L. T. Hoang, N. J. Cox, and J. M. Katz. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J. Virol. 79:4201–4212. 2005.

22. Perkins, L. E., and D. E. Swayne. Comparative susceptibility of selected avian and mammalian species to a Hong Kong-origin H5N1 high-pathogenicity avian influenza virus. Avian Dis. 47:956–967. 2003.

23. Perkins, L. E., and D. E. Swayne. Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet. Pathol. 38:149–164. 2001.

24. Perkins, L. E., and D. E. Swayne. Pathogenicity of a Hong Kong–origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. Avian Dis. 46:53–63. 2002.

25. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497. 1938.

26. Shortridge, K. F., N. N. Zhou, Y. Guan, P. Gao, T. Ito, Y. Kawaoka, S. Kodihalli, S. Krauss, D. Markwell, K. G. Murti, M. Norwood, D. Senne, L. Sims, A. Takada, and R. G. Webster. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 252:331–342. 1998.

27. Songserm, T., R. Jam-on, N. Sae-Heng, N. Meemak, D. J. Hulse-Post, K. M. Sturm-Ramirez, and R. G. Webster. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerg. Infect. Dis. 12:575–581. 2006.

28. Stallknecht, D. E. Ecology and epidemiology of avian influenza viruses in wild bird populations: waterfowl, shorebirds, pelicans, cormorants, etc. In: Proc. Fourth International Symposium on Avian Influenza. D. E. Swayne and R. D. Slemons, eds. American Association of Avian Pathologists. Kennett Square, PA. pp. 61–69. 1998.

29. Sturm-Ramirez, K. M., T. Ellis, B. Bousfield, L. Bissett, K. Dyrting, J. E. Rehg, L. Poon, Y. Guan, M. Peiris, and R. G. Webster. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J. Virol. 78:4892–4901. 2004.

30. Sturm-Ramirez, K. M., D. J. Hulse-Post, E. A. Govorkova, J. Humberd, P. Seiler, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. Chaisingh, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J. Virol. 79:11269–11279. 2005.

31. Swayne, D. E. Pathobiology of H5N2 Mexican avian influenza virus infections of chickens. Vet. Pathol. 34:557–567. 1997.

32. Swayne, D. E., and M. Pantin-Jackwood. Pathogenicity of avian influenza viruses in poultry. Dev. Biol. (Basel) 124:61–67. 2006.

33. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian influenza. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. J. R. Glisson, D. E. Swayne, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennet Square, PA. pp. 150–155. 1998.

34. Swayne, D. E., and D. L. Suarez. Highly pathogenic avian influenza. Rev. Sci. Tech. Off. Int. Epizoot. 19:463–482. 2000.

35. Tumpey, T. M., D. L. Suarez, L. E. Perkins, D. A. Senne, J. G. Lee, Y. J. Lee, I. P. Mo, H. W. Sung, and D. E. Swayne. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. J. Virol. 76:6344–6355. 2002.

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